Cellular response to dental monomers

Involvement of oxidative stress and DNA damage

Linda Haugland Evensen

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Department of Biosciences

Faculty of Mathematics and Natural Sciences

University of Oslo

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Linda Evensen

Abstract

Polymer-based composite materials have replaced the use of amalgam as filling material of choice in modern dentistry. Methacrylate monomers are key constituents in the polymer-based composite materials. The monomers are found to be airborne in dental practices, making airway exposure likely. Human bronchial epithelial cells, BEAS-2B, were therefore used as an *in vitro* model system.

Methacrylate monomers have been associated with cell growth disturbances, glutathione depletion, DNA damage and cell death in different human cell lines. The detailed cellular mechanisms underlying these effects are still unclear. Oxidative stress caused by glutathione depletion is suggested as a possible mechanism in the development of cytotoxic effects. However, resent findings indicate other mechanisms to be of importance.

In the current study, the mechanisms involved in the cellular response to the two commonly used methacrylate monomers glycerol dimethacrylate (GDMA) and 2-hydroxyethyl methacrylate (HEMA) were investigated. The main focus was to map DNA damage response and the involvement of glutathione depletion. In order to investigate the role of glutathione depletion and oxidative stress, the effect of the monomers were compared to two glutathione depleting agents, diethyl maleate (DEM) and buthionine sulfoximine (BSO).

Glutathione depletion, oxidative stress, cell cycle disturbances and DNA damage were observed in BEAS-2B cells following exposure to both GDMA and HEMA. Similar effects on glutathione depletion and increased oxidative stress were seen in the two exposure scenarios. The effects on cell growth, cyclin levels and activation of DNA damage signalling proteins differed between the monomers, indicating differences in cellular response mechanisms. The results show that oxidative stress caused by glutathione depletion cannot alone explain the monomer-induced cytotoxicity *in vitro*.

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1 Introduction

1.1 Biomaterials

Biomaterials are synthetic materials used for replacement of a part or a function of the body, with the ultimate goal of improving human heath [1]. Disease or trauma may cause the need for artificial heart valves, cochlear implants, joint replacements or dental restorations. These are all some examples of biomaterials used in medicine today.

The important challenge regarding biomaterials is that they are designed to replace living tissue that has had millions of years of evolution to adapt to their unique role and function [2]. Biomaterials need therefore to be structurally and functionally compatible and function as a substitute for the body part it has replaced.

The term biocompatibility refers to a materials ability to perform its desired functions, without causing unwanted biological effects in the recipient [3]. An evaluation of biocompatibility includes a number of scientific fields including cell biology, toxicology, medicine and physics. The potential risk of unwanted biological effects when using biomaterials makes biocompatibility related to risk assessment [4]. The challenge is to develop biomaterials with qualities that outweigh the potential risks. The precautionary principle and the risk of health effects make the field of biomaterials dynamic with a constant potential for improvements. Research on the biological effects of these materials therefore results in improvement of their quality and lead to progression of the field of biomaterials.

1.2 Dental biomaterials

For about 150 years, the restorative material amalgam has been the dental filling material of choice, and in some parts of the world, it still is [5]. Amalgam is a metal alloy consisting of about 50% mercury (Hg) in addition to silver (Ag), tin (Sn) and copper (Cu) [6]. Mercury can be very toxic to humans, affecting the central nervous system, the gastrointestinal tract and the respiratory system [7]. It is also considered an environmental pollutant [8]. Because it is nonbiodegradable it

can potentially bioaccumulate in aquatic and terrestrial organisms once released into the environment.

It was the growing concern about mercury as an environmental pollutant that made Norway issue a ban of all the use, production and import of mercury in 2008 [9]. Amalgam is affected by this ban, and can therefore no longer be used as a dental restorative material. A more esthetical alternative to amalgam is the polymer-based composite filling materials [5]. In general, composite materials are composed of two or more components to produce a product that has properties exceeding the individual components on their own [4].

Polymer-based composite filling materials consists of three main components [4], a polymer matrix, filler particles, and coupling agents. The polymer matrix is composed of methacrylate monomers bound together to form strong polymer structures. The polymer matrix is reinforced with filler particles to increase physical and mechanical properties of the material. To approve the binding between the polymer matrix and the filler particles, coupling agents are used. Polymer-based composite materials comprise several types of filling materials such as compomeres, adhesives and polymer-based glassionomers. For simplicity, these materials are referred to as composite filling materials throughout this thesis.

1.3 Chemistry of methacrylate monomers

A polymer is a large molecule composed of repeated units called monomers bound together in chains and by cross-linking (fig. 1). To form this polymer matrix, monomers are linked together in a polymerization reaction. Methacrylate monomers such as glycerol dimethacrylate (GDMA) and 2-hydroxyethyl methacrylate (HEMA) (fig. 2) are used as precursors to form the polymer matrix in composite filling materials. The reactive part of the monomer is the electrophilic carbon resulting from the carbon-carbon double bond in the carboxylic ester group (methacrylate group). HEMA, being a mono-methacrylate, have one methacrylate group (one electrophilic carbon), while GDMA is a di-methacrylate and have two methacrylate groups (two electrophilic carbons).

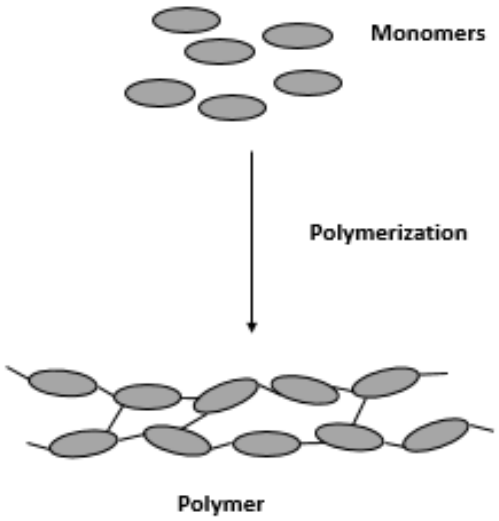

Figure 1. Illustration of the polymerization process

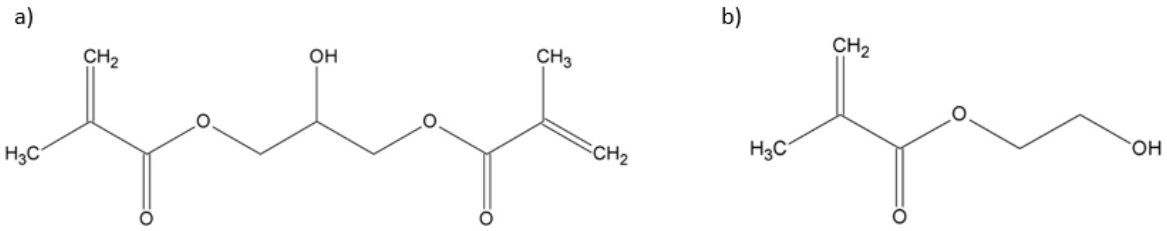

Figure 2. a) Glycerol dimethacrylate (GDMA) and b) 2-hydroxyethyl methacrylate (HEMA)

The polymerization reaction is dependent on monomers ability to form free radicals [4]**.** To start the reaction a free radical is needed as an initiator, this can be introduced by chemical agents, heat or via light activation. The free radical can react with a monomer molecule by extracting an electron from their double bond, leaving the other electron of the double bond unpaired. This forms a new free radical site in the molecule, and the reaction is initiated. The reaction continues adding monomers to the growing polymer chain, until the reaction is terminated. Termination can occur because of a combination between two growing polymer chains or the transfer of a hydrogen atom to another polymer.

Methacrylate monomers have different chemical properties, but they all have at least one carboxylic ester group (methacrylate group), conjugated with a double bond between the α- and the β-carbon. This is the reactive part of the molecule during polymerization. The double bond between the two carbon atoms results in an electrophilic β-carbon [10, 11]. Electrophilic chemicals can react and bind to endogenous molecules that have a nucleophilic carbon such as glutathione, DNA and proteins.

The degree of conversion reflects the percentage of methacrylate double bonds that have reacted during polymerization [4]. The degree of conversion rarely exceeds 75 % [12]. This can result in release of unpolymerized monomers after placement of the dental materials [13].

1.4 Exposure to methacrylate monomers

Both dental personnel and patients are exposed to components of dental materials [13-17], but the extent and the severity is not fully known. Major absorption routes for foreign chemicals are through the skin, gastrointestinal tract and the lungs, depending on the nature of the chemical and the exposure situation.

After restorative treatment, measurable amounts of methacrylate monomers have been detected in salvia of the patients [13], where the monomers can interact locally or reach the gastrointestinal tract. Monomers are also present in the air of dental practices [15, 16], making exposure through the lungs a possible absorption route for both personnel and patients during the handling of composite filling materials. Upon inhalation, chemicals can diffuse into the bloodstream. Compounds that reach the bloodstream can then be transported rapidly throughout the body [4, 7].

The skin is the largest organ in the human body and functions as a relatively impermeable barrier against foreign substances. Many chemicals can despite of this penetrate the skin and cause local and possibly systemic effects. Published studies on allergic contact dermatitis and allergy in dental personnel [18, 19], demonstrates that methacrylate monomers can be absorbed through the skin and cause allergic effects. Due to degradation of the materials over time, long-term exposure may also be of relevance for the *in vivo* situation [17].

1.5 Toxic responses

The biological responses to foreign chemicals are many and variable. Chemical structure and properties, exposure dose and duration will influence the response. Toxicity can be local or systemic, reversible or irreversible, immediate or delayed [20].

Development of toxicity usually involves a reaction between the ultimate toxicant and a target molecule [20]. By interacting with molecules with different roles, the toxicant produces effects that can impair several functions in a cell. Insufficient repair or inappropriate adaptation to damaging agents can lead to inflammation, DNA damage, dysregulation of the cell cycle and cell death [7].

1.5.1 Cell death

There are two main morphological changes seen in dying cells (fig. 3); apoptotic cells undergo shrinkage and condensation of the chromosomes, while necrotic cells swell and rupture, causing inflammation and damage to the surrounding tissue [7]. Apoptosis is a form of programmed cell death, where the cell initiates a process that eventually leads to the phagocytosis of the damaged cell by macrophages and other phagocytes [21]. This prevents damage to the surrounding tissue.

Apoptosis is a natural part of the maintenance and renewal of tissues, but it can also be a response to a toxicant. More acute damage to cells might result in necrosis. Necrotic cell death can be described as a chaotic process characterized by the rupture of membranes, dissolution of organelles and loss of homeostasis and ATP [7]. Some toxicants have a tendency to induce apoptosis at low concentrations, but when the dose and exposure time increases, the cells become energy depleted and necrosis is the only possible outcome [20, 22].

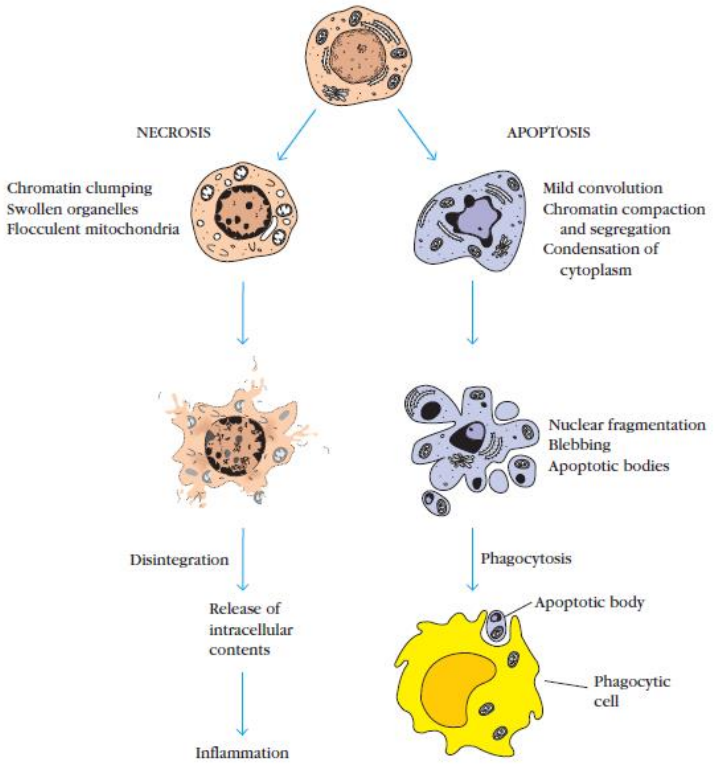

Figure 3. Morphological changes seen in cells during apoptotic and necrotic cell death. Figure from [23]

1.5.2 Oxidative stress

Reactive oxygen species (ROS) are free radicals containing oxygen atoms, like superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO^{\bullet}$). They are highly reactive because of their unpaired electrons. ROS are natural by-products of oxygen metabolism, but they are toxic to cells if present in excessive amounts. Toxicants are also able to increase levels of ROS in cells, directly or indirectly [20].

To protect itself from the damaging effects of ROS, cells have protective molecules called antioxidants. Ascorbic acid (vitamin C), carotenes (β-carotene, retinol) and glutathione (GSH) are all antioxidants that protect cells from oxidative damage. GSH is an important antioxidant in the human defence system, and is found in most cells with the highest concentration occurring in the liver (5mM or more) [7], reflecting this organs role in detoxification of toxicants. If a toxic

substance depletes the cells of its GSH content, it can lead to increased levels of ROS, this situation is known as oxidative stress (fig. 4) [7]. Oxidative stress can lead to lipid peroxidation, protein damage and DNA strand breaks, cell injury and/or cell death [20].

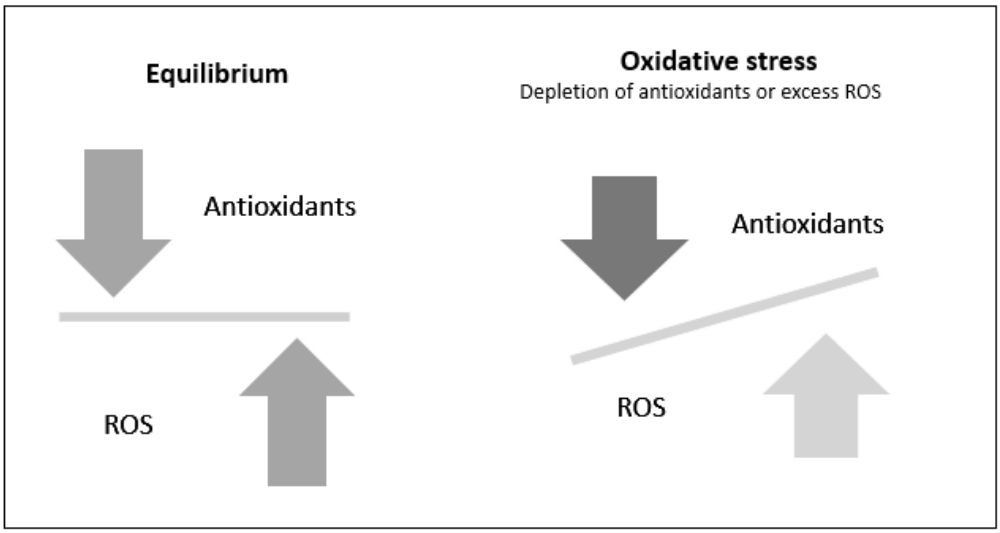

Figure 4. Oxidative stress is associated with a depletion of antioxidants or an excess of ROS

In addition to being an abundant antioxidant, glutathione also have a role in metabolism of toxicants (detoxification). The goal is to reduce toxicity and facilitate excretion. In phase 1 metabolism, a polar or reactive chemical group is added to toxicants by enzymes such as the cytochrome P450 oxidases [7]. Phase 2 metabolism involves conjugation between endogenous chemicals and toxicant. Glutathione conjugation is an important phase 2 reaction in detoxification [7], both due to the reactivity towards electrophilic molecules and because of the high concentrations of glutathione in cells. Because of the nucleophilic SH-group, it can react with electrophilic molecules making them more polar (and more water-soluble) which will facilitate excretion.

1.5.3 DNA damage and cell cycle regulation

The DNA molecule contains all the genetic information in organisms, and encodes all the molecules that are synthesized in the cells. DNA damage is any kind of change in the DNA molecule and can be the result of exposure to different chemicals and radiation. When a cell divides, it copies its genome, and passes one copy to each of the two daughter cells that are produced. DNA damage that is not repaired can result in mutations that can lead to genetic diseases because of dysfunctional or non-functional proteins. Mutations can ultimately lead to the formation of a tumour that can develop into cancer. The number and severity of inheritable human diseases that have been linked with defects in DNA repair processes, show how important it is for cells to repair any damaged DNA [24].

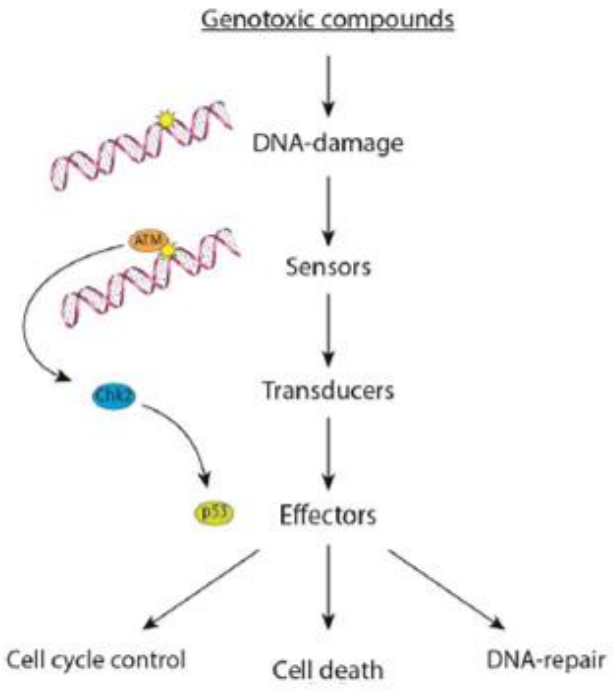

Figure 5. DNA damage starts signalling cascades that eventually results in cell cycle control, cell death or DNA-repair. Modified figure from PhD thesis by Ansteinsson [25]

Sensor proteins guarding the DNA can recognize damage and initiate signalling pathways via transducers to effector proteins (fig. 5). The effector proteins activate DNA repair processes or eliminate the cell via apoptosis if the damage is beyond repair [26]. One of the more serious forms of DNA damage is a double strand break, because this will destroy the double helical structure of the DNA molecule, making DNA repair more difficult. Single strand breaks are not as critical because the double helix stays intact [24, 27]. The cell response to double strand breaks involves activation of signalling cascades via the sensor proteins ataxia telangiectasia mutated (ATM) [27] and H2AX (activated form is known as γH2AX) [28]. ATM further activates, via phosphorylation, several transducer and effector proteins that can regulate DNA damage checkpoints in the cell cycle. Proteins that are phosphorylated as a part of this signalling cascade include P53 [29] and Chk2 [30]. P53 has multiple roles in cell signalling and is activated by several stressors. Different forms of DNA damage are able to activate P53, which leads to an increase in cell concentration of pP53 that is proportional to the amount of DNA damage [29].

DNA damage checkpoints ensure that a cell enters some form of cell cycle arrest to repair damaged DNA before replication and cell division. Cyclin-dependent kinases (CDKs) and proteins known as cyclins regulate progression through the cell cycle. The CDKs are catalytic subunits of an effective enzyme, and the enzyme is not active unless bound to the regulatory subunit, the cyclins. The CDKs activity is controlled through phosphorylation, by inhibitory proteins and by the availability of the cyclins [31].

1.6 Toxicity of methacrylate monomers

Both *in vivo* and *in vitro* studies have identified toxic responses to composite filling materials [32]. Dental personnel are known to suffer occupational dermatitis and skin diseases following contact with reactive chemicals from dental materials [33, 34]. It has also been rapports on contact allergy and dermatitis related specially to methacrylate monomer exposure [18, 19].

Most studies on the toxicity of methacrylate monomers are conducted *in vitro,* and one of the most studied monomer is HEMA. Methacrylate monomers are cytotoxic and they are able to induce cell death in different human cell lines [35-39]. Cell cycle arrest and DNA damage is indicated after *in vitro* exposure to HEMA [40, 41]. The mechanisms behind the cytotoxicity are however unclear.

Adduct formation with glutathione (GSH) have been suggested as a main mechanism in methacrylate monomer induced cytotoxicity [36, 37, 39, 42]. Both GDMA and HEMA have been shown to form adducts with and thereby deplete the cellular content of GSH in cells [10, 43]. This will lower the cells ability to counteract oxidative damage, and possibly lead to oxidative stress. Oxidative stress can then be the initial event causing apoptosis, cell cycle arrest and DNA damage. The monomers ability to deplete GSH seem, however, not to correspond to their cytotoxic potential [10]. Additional mechanisms are therefore likely to be involved in the cellular response to methacrylate monomers.

1.7 Aims

The working hypothesis of this study was that the methacrylate monomers GDMA and HEMA induce DNA damage by different cellular mechanisms.

The specific aim was to study and compare cellular mechanisms in response to GDMA and HEMA exposure. This involved to:

- Investigate glutathione and oxidative stress in the cellular response after exposure
- Investigate DNA damage signalling pathways and responses after exposure

2 Materials

2.1 Chemicals and antibodies

Table 1. List of chemicals

Chemicals (CAS no.)	Product number	Manufacturer
2-Hydroxyethyl methacrylate (HEMA) (868-77-9)	64166	Sigma-Aldrich, St. Louis, USA
2,7-dichlorofluorescein diacetate (DCFH-DA) (4091-99-0)	D6883	Sigma-Aldrich, St. Louis, USA
2-Mercaptoethanol (60-24-2)	M6250	Sigma-Aldrich, St. Louis, USA
30 % Acrylamide/Bis Solution	161-0158	Bio-Rad Laboratories, Inc., CA, USA
Ammonium persulfate (APS) (7727-54-0)	A3678	Sigma-Aldrich, St. Louis, USA
Annexin V-FITC Apoptosis Detection Kit	BMS500FI	Bender MedSystems GmbH, Vienna, Austria
Bovine collagen solution (9007-34-5)	5005-B	Advanced Biomatrix, INAMED corp., Fremont, USA
Bovine serum albumin (BSA) (9048-46-8)	A7906	Sigma-Aldrich, St. Louis, USA
Bromophenol blue sodium salt (62625-28-9)	B8026	Sigma-Aldrich, St. Louis, USA
Buthionine sulfoximine (BSO) (83730-53-4)	B2515	Sigma-Aldrich, St. Louis, USA
Diethyl maleate (DEM) (141-05-9)	W505005	Sigma-Aldrich, St. Louis, USA
Dimethyl sulphoxide (DMSO) (67-68-5)	116743	Merck KGaA, Darmstadt, Germany
Fetal bovine serum (FBS)	F4135	Sigma-Aldrich, St. Louis, USA
Gibco LHC-9 cell medium	12680-013	Invitrogen-Life Technologies, CA, USA
Glycerol 85% (56-81-5)	104094	Merck KGaA, Darmstadt, Germany
Glycerol-dimethacrylate (GDMA) (1830-78-0)	436895	Sigma-Aldrich, St. Louis, USA
Glycine (56-40-6)	G7126	Sigma-Aldrich, St. Louis, USA
Hepes Buffered Saline Solution (HEPES buffer)	CC-5024	Lonza, Verviers, Belgium
Hoechst 33342 (23491-52-3)	B2261	Sigma-Aldrich, St. Louis, USA
Methanol (67-56-1)	106007	Merck KGaA, Darmstadt, Germany
Molecular weight marker	928-40000	LiCor Biosciences, Hamburg, Germany
Monobromobimane (71418-44-5)	69898	Sigma-Aldrich, St. Louis, USA
MTT solution (Thiazolyl Blue Tetrazolium Bromide) (298-93-1)	M2128	Sigma-Aldrich, St. Louis, USA
N-Ethylmaleimide (NEM) (128-53-0)	E3876	Sigma-Aldrich, St. Louis, USA
Nuclear isolations and staining solution (NIM-DAPI)	731085	NPE Systems, Florida, USA
Phosphate buffered saline (PBS)	BE17-516F	Lonza, Verviers, Belgium
Ponceau S (6226-79-5)	P-3504	Sigma-Aldrich, St. Louis, USA

Propidium iodide solution (PI) (25535-16-4)	70335	Sigma-Aldrich, St. Louis, USA
Sodium chloride (NaCl) (7647-14-5)	S5886	Sigma-Aldrich, St. Louis, USA
Sodium dodecyl sulfate (SDS) (151-21-3)	L4390	Sigma-Aldrich, St. Louis, USA
Sucrose (57-50-1)	10274	Analar, BDH Middle East LLC, Dubai
Tetramethylethylenediamine (TEMED) (110-18-9)	T9281	Sigma-Aldrich, St. Louis, USA
Tricine (5704-04-1)	T5816	Sigma-Aldrich, St. Louis, USA
tri-Natriumcitrat-2-hydrat (tri-sodiumcitrat) (6132-04-3)	106448	Merck KGaA, Darmstadt, Germany
Trizma base (77-86-1)	T1503	Sigma-Aldrich, St. Louis, USA
Trypsin/EDTA solution (9002-07-7)	CC-5012	Lonza, Verviers, Belgium
Tween 20 (9005-64-5)	822184	Merck KGaA, Darmstadt, Germany
Virkon	1232-8667	Antec Int.-A Du Pont Comp., Suffolk, UK

Table 2. Antibodies

Antibodies	**Product number**	**Manufacturer**
β-actin	4967	Cell signaling Technology, Inc., USA
Phospho-p53 (Ser15)	9284	Cell signaling Technology, Inc., USA
Phospho-Chk2 (Thr68)	2661	Cell signaling Technology, Inc., USA
Phospho-Histone H2A.X (Ser139)	2577	Cell signaling Technology, Inc., USA
Cyclin D1 (92G2)	2978	Cell signaling Technology, Inc., USA
Cyclin E1 (HE12)	4129	Cell signaling Technology, Inc., USA
Goat anti-mouse IgG, secondary Ab	926-32210	LiCor Biosciences, Hamburg, Germany
Goat anti-rabbit IgG, secondary Ab	926-68071	LiCor Biosciences, Hamburg, Germany

2.2 Equipment and software

Table 3. Equipment and software

Equipment and software	**Manufacturer**
Accuri C6 Flow Cytometer	BD Bioscience, Bedford, MA, USA
Aerosol Resistant Tips (ART) pipette tips	Molecular Bio Products, USA
Autoclave (HV-50L)	Hirayama, Hong Kong
Bürker counting chamber	Assistant, Sondheim, Germany
Cell culture flasks	BD Falcon, Bedford, MA, USA
Cell Lab Quanta SC Flow Cytometer	Beckman Coulter, Florida, USA

CO_2 incubator (SANYO MCO-18AIC(UV))	Surplus Solutions, LLC, USA
Costar plates	Corning Incorporated, NY, USA
Electrophoresis systems and blotting module	TetraCell, Bio-Rad Inc., CA, USA
GraphPad Prism 4 software	GraphPad Software, CA, USA
Image Studio software	LiCor Biosciences, Hamburg, Germany
Micropipettes	Sarstedt, Nümbrecht, Germany
Multicycle	Phoenics Flow Systems, CA, USA
Odyssey CLx Western blot scanner	LiCor Biosciences, Hamburg, Germany
Olympus BX51 fluorescence microscope	Olympus Europe, Hamburg, Germany
Olympus C7070 camera	Olympus Europe, Hamburg, Germany
Olympus CKX41 inverted phase contrast microscope	Olympus Europe, Hamburg, Germany
Olympus DP70 camera	Olympus Europe, Hamburg, Germany
Rotina 35R Centrifuge	Hettich, Tuttlingen, Germany
SensION pH31, pH meter	Hach Company, Colorado, USA
Synergy H1 Spectrophotometric plate reader	BioTek Instruments Inc., Vermont, USA

2.3 Buffers and gels

Table 4. Sample buffer, gel buffer and TBS buffer

Chemicals	**Sample buffer**	**Gel buffer**	**5x TBS buffer**
Trizma base	1.817 g	90.75 g	30.3 g
Glycine			
Glycerol 85 %	30 g		
SDS	12 g		
NaCl			43.8 g
Distilled water		250 mL	1000 mL
pH	Adjust to 7.0	Adjust to 8.46	Adjust to 7.5
	Adjust final volume to 100 mL with dest.water		

Table 5. Anode buffer, cathode buffer and transfer buffer

Chemicals	**10x Anode-buffer**	**10x Cathode-buffer**	**10x Transfer buffer**
Trizma base	60.57 g	60.57 g	30.3 g
Glycine			144.1 g
Tricine		89.59 g	
SDS		5 g	
Distilled water	500 mL	500 mL	800 mL
pH	Adjust to 8.9	Adjust to 8.25	Do not adjust!
			Adjust final volume to 1000 mL with dest.water

Table 6. Separating gel and stacking gel

Chemicals	**Tricine separating gel (16 %)**	**Stacking gel (4 %)**
30 % Acrylamide/Bis solution	16 mL	1.6 mL
Gel buffer	10 mL	3 mL
Glycerol 85 %	3.5 g	
Distilled water	Ad 30 mL	Ad 12 mL
10 % SDS	300 µL	90 µL
10 % APS	100 µL	90 µL
TEMED	10 µL	10 µL

Table 7. Citrate buffer

Chemicals	**Citrate buffer**
Sucrose	85.5 g
Tri-sodiumcitrate	11.8 g
DMSO	50 mL
Distilled water	Ad 1000 mL

3 Methods

3.1 Cell line

Human bronchial epithelial cells, BEAS-2B, is an adenovirus (12-SV40 hybrid) transformed cell line [44]. The cell line is available from European Tissue Type Culture Collection (ECACC) and distributed by Sigma-Aldrich. The cells are cultured in serum-free cell culture medium, LHC-9, formulated for the growth of bronchial epithelial cells *in vitro.* BEAS-2B is classified as a biosafety level 2 cell line (Material safety sheet, 2009).

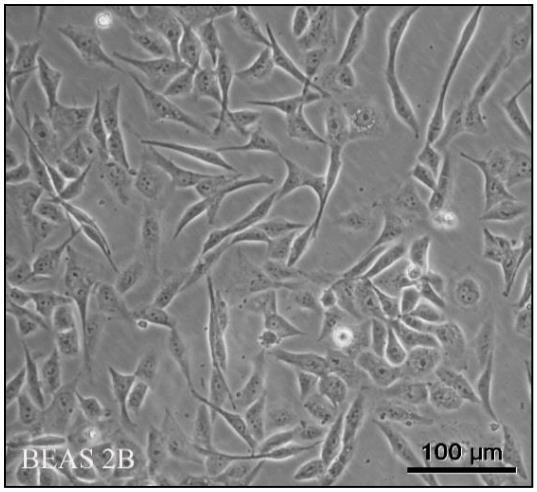

Figure 6. Phase contrast microscopy picture of BEAS-2B cells

3.1.1 Treatment of cells

Cells were cultivated in sterile cell culture flasks and kept in an incubator under stable conditions (37 °C, 5 % CO_2, > 95 % relative humidity). BEAS-2B is an adherent cell line. Culture flasks and -plates were pre-coated with collagen (30 µg/ml in Hepes buffer) before plating the cells. The cells were monitored daily by phase contrast microscopy. When the cells were 80 – 85 % confluent, they were diluted 1:5 and transferred to a new flask for further growth. Medium was changed 24 hours after dilution. Trypsin was used to detach cells from the coating surface. Trypsin is a proteolytic enzyme, which breaks down the proteins that binds the cells to the surface, and dissociates adherent cells from the coating. Medium, trypsin and other solutions were preheated to 37 °C before added to the cells.

Cells that were not used were discarded in 2 % Virkon solution. Equipment that had been in contact with the cells was sterilized using an autoclave (121 °C, 60 min).

3.1.2 Seeding and exposure of cells

Twenty-four hours before the start of an experiment, cells were counted in a Bürker counting chamber and transferred onto pre-coated plates for cell growth (25 000 cells/cm²). Medium was changed one hour before the cells were exposed.

Stock solutions of GDMA (2 mM), HEMA (200 mM), buthionine sulfoximine (BSO) (2.5 mM) and diethyl maleate (DEM) (6 mM) were prepared fresh before every experiment by diluting the respective substances in cell medium. A calculated volume of the stock solutions was added to the cell culture to obtain the final exposure concentrations.

3.2 MTT assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay measures the activity of succinate-dehydrogenase (SDH) enzymes in cells [45]. SDH-enzymes in living cells reduce the yellow MTT substrate to a blue formazan product (fig. 7). The amount of formazan product therefore reflects the SDH-activity and viability in the cell cultures. The SDH-activity is assessed by measuring absorption using a spectrophotometer at a wavelength of 570 nm. MTT is a commonly used assay to assess cell viability in cytotoxicity studies, and is applied to many different cell cultures [46-48].

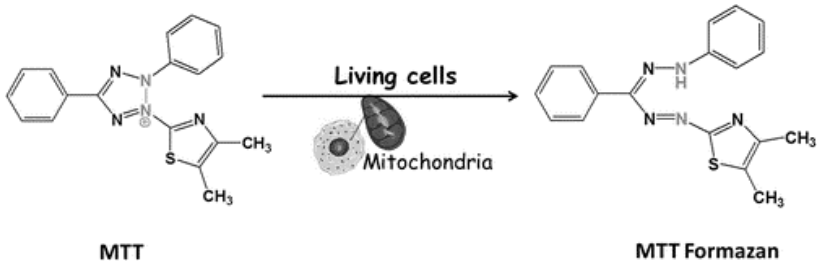

Figure 7. MTT is reduced to formazan by mitochondrial SDH-enzymes in living cells. Figure from Mao Z et al. [49]

Procedure:

- Medium is replaced with 500 µl MTT solution (0.5 mg/ml PBS)
- 1 hour incubation (37 ˚C, 5 % CO_2, > 95 % relative humidity)
- MTT solution is removed
- 500 µl dimethyl sulphoxide (DMSO) is added to dissolve the formazan
- The plate is shaken for 10 min
- Absorbance at 570 nm is red on a spectrophotometer

3.3 Fluorescence microscopy

Changes in cellular morphology can be visualized by staining cellular compartments with fluorochromes and looking at the cells in a fluorescence microscope. Hoechst 33342 and propidium iodide (PI) are fluorochromes that bind DNA. Hoechst 33342 can penetrate intact cell membranes and gives blue fluorescence, while PI does not penetrate cell membranes and gives red fluorescence. Viable cells will be stained blue by Hoechst 33342 evenly throughout the nucleus. Apoptotic cells will be stained more intense with Hoechst 33342, because of their condensed chromosomes. Necrotic cells will lack membrane integrity and therefore be stained red with PI.

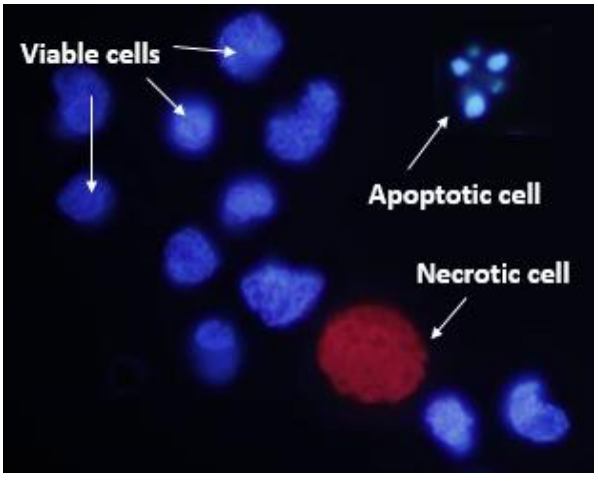

Figure 8. Fluorescence microscopy picture of typical viable, apoptotic and necrotic cells stained with Hoechst 33342 and PI

Procedure:

- The cells and medium are centrifuged (250 g, 4 ˚C, 10 min) and the supernatant is removed
- 30 minutes incubation in room temperature (protected from light) with 50 µl FBS with Hoechst 33342 (10 µg/ml) and PI (5 µg/ml)
- One drop of the cell solution is spread out on a microscope slide. The slide is left to dry in the dark
- The slide is studied in a fluorescence microscope (excitation filter 340 – 380 nm)
- 300 cells are counted in each sample

3.4 Flow cytometry

Flow cytometry is a technique used to analyse characteristics of individual cells. A flow cytometer obtains information about each cell by measuring light scatter and fluorescence that the cells emit as they flow through a light source, usually a laser or a UV-lamp [50]. Information such as DNA content, cell size, viability and cell death on a single cell level can be obtained. In addition, this technic can be used to sort cells in a mixture based on different cell surface markers.

3.4.1 Cell death analysis

Apoptotic cell death leads to the translocation of phosphatidylserine (PS) from the inner to the outer side of the plasma membrane of eukaryotic cells. Annexin V is a protein with high affinity for PS. In the early stages of apoptosis, the cell membrane stays intact, while cells that undergo necrosis will lose their membrane integrity. Annexin V will therefore bind apoptotic cells. Measurements of annexin V binding, simultaneously with a dye exclusion test to assess the membrane state, will give results discriminating between apoptotic and necrotic cells [51]. Propidium iodide (PI) staining can be used to test the membrane state, as PI cannot penetrate intact cell membranes.

Procedure:

- Cells and medium are and centrifuged (250 g, 4 ˚C, 10 min), washed with 0.5 mL PBS and centrifuged again.
- 100 µl of binding buffer with annexin V (20 µl/ml) and PI (20 µl/ml) is added and mixed with the cell pellet. (Binding buffer, annexin V and PI from Annexin V-FITC Apoptosis Detection Kit). Incubation for 10 min protected from light.
- 400 µl of binding buffer is added to increase the test volume
- Annexin V and PI binding are measured on a flow cytometer

3.4.2 Cell cycle analysis

The eukaryotic cell cycle consists of four stages; G1, S, G2 and M. During S (synthesis) phase, the DNA is replicated. The M-phase (mitosis) is the cellular division process where the chromosomes are separated to produce two daughter cells. During the gap phases (G1 and G2) the cells grow and synthesise proteins. Through G1, S and G2 the DNA content in the cells therefore vary, depending on the cells progression in the cell cycle. A cell in late S phase and G2 will have twice the amount of DNA as a cell that is growing in G1. By measuring the amount of DNA present in each cell, the stage in the cell cycle can be determined (fig. 9). The reagent NIM-DAPI (4',6-diamidino-2-phenylindole-dihyrochloride) binds to DNA and gives fluorescence that can be measured on a flow cytometer. The intensity of the fluorescence will then be proportional to the amount of DNA in the cell [52].

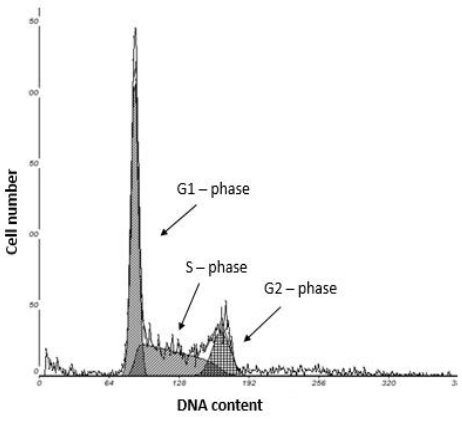

Figure 9. DNA histogram from Multicycle software. DNA content is measured and used to determine the stage in the cell cycle

Procedure:

- Cells are washed with PBS and incubated with trypsin
- 600 µl PBS with 10 % FBS are added to the cells
- The cell suspension is centrifuged (250 g, 4 ˚C, 10 min) and the supernatant is removed
- 100 µl citrate buffer is added and mixed with the cell pellet
- The samples is stored for minimum 24 hours in the freezer (-20 ˚C)
- Samples are incubated and mixed with 300 µl NIM-DAPI solution (in the dark) for 10 minutes before analysing
- Fluorescence are measured on a flow cytometer
- Results are further analysed in Multicycle software

3.4.3 Measurement of glutathione (GSH)

Glutathione (GSH) concentration in cells can be measured by flow cytometry using the reagent monobromobimane [53]. Monobromobimane pass through cell membranes and bind to the sulfhydryl (SH) group in GSH, forming fluorescent adducts. GSH level in the cells can then be assessed by measuring the fluorescence with a flow cytometer. Background fluorescence was determined by measuring the fluorescence of cells depleted of GSH after incubation with N-Ethyl maleimide (NEM).

Procedure:

- Cells are washed with PBS and treated with trypsin
- 400 µl PBS with 10 % FBS and 40 µM monobromobimane is added to the cells
- 15 min incubation protected from light (room temperature)
- Fluorescence is measured on a flow cytometer
- Results are adjusted according to background fluorescence (NEM treated cells), and presented as present of control

3.4.4 Measurement of reactive oxygen species (ROS)

The amount reactive oxygen species (ROS) in cells can be measured on a flow cytometer using the reagent DCFH-DA (2,7-dichlorodihydrofluorescein diacetate). Cellular esterases convert DCFH-DA to the polar molecule DCFH that is further oxidized to DCF by intracellular ROS. DCF gives fluorescence that can be detected using a flow cytometer. The level of DCF in the cells will be proportional to the amount of ROS [54].

Procedure:

- Cells are incubated with 10 µl DCFH-DA (20 µM) the last 15 minutes of the exposure time
- The cells are treated with trypsin and harvested using 400 µl PBS with 10 % FBS
- Fluorescence is measured on a flow cytometer
- Fluorescence in control cells is set to 100 % and the amount of ROS in the exposed cells is calculated as percent of control

3.5 Western blotting

Western blotting is a comprehensive technique used to detect and quantify proteins. It is well explained in literature and an important technique in cell biology [50, 55, 56]. Proteins are extracted from cells and separated based on molecular weight through gel electrophoresis. Gel electrophoresis uses an electric field to arrange proteins according to their size; the smaller the proteins are the longer they will travel through the pores in the gel. By loading a protein marker in the gel, the molecular weight of the proteins can be determined. After separation on the gel, the proteins are transferred to and immobilized on a nitrocellulose membrane. The transfer is done using an electric field; the negatively charged proteins will move towards the positive field, were the nitrocellulose membrane is placed. The membrane is treated with 5 % bovine serum albumin (BSA) to prevent nonspecific binding of antibody. Further, it is incubated with a primary antibody specific to the protein in question, then with a secondary antibody that binds to the primary antibody and allows visualization of the proteins (fig. 10).

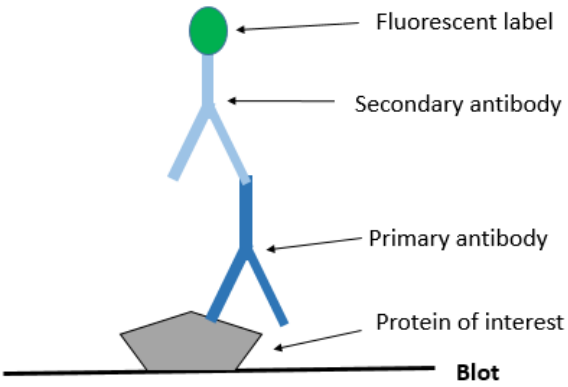

Figure 10. Illustration of how proteins are detected by the use of antibodies and fluorescent label in western blotting

Procedure:

- Cells are washed with PBS and harvested using a cell scrape and 130 µl sample buffer
- 15 µl of each sample (total protein of 30 µg) is electrophoresed and blotted onto a nitrocellulose membrane
- Membrane is stained with Ponceau S for loading control
- The membrane is blocked in 5 % BSA in TBS-T (TRIS-buffered saline with 0.1 % Tween) for 20 minutes
- Incubation with primary antibody diluted (1:2500) in 1 % BSA in TBS-T at 8 ˚C overnight
- The membrane is washed three times (15 min) in TBS-T
- Incubation with secondary antibody diluted (1:10 000) in 1 % BSA in TBS-T for 2 hours at room temperature
- Membranes are scanned and the results analysed in Image Studio software

3.6 Statistics

P-values are calculated using a student t-test in Microsoft Office Excel; $p < 0.05$ are considered significant. Graphs are made in Graph Pad prism 4.

4 Results

4.1 Cell viability

The MTT assay measures SDH-activity and was used to estimate cell viability after 24 hours of exposure to GDMA and HEMA. Significant reduction in cell viability was observed after exposure to concentrations equal to or higher than 0.2 mM GDMA and 4 mM HEMA.

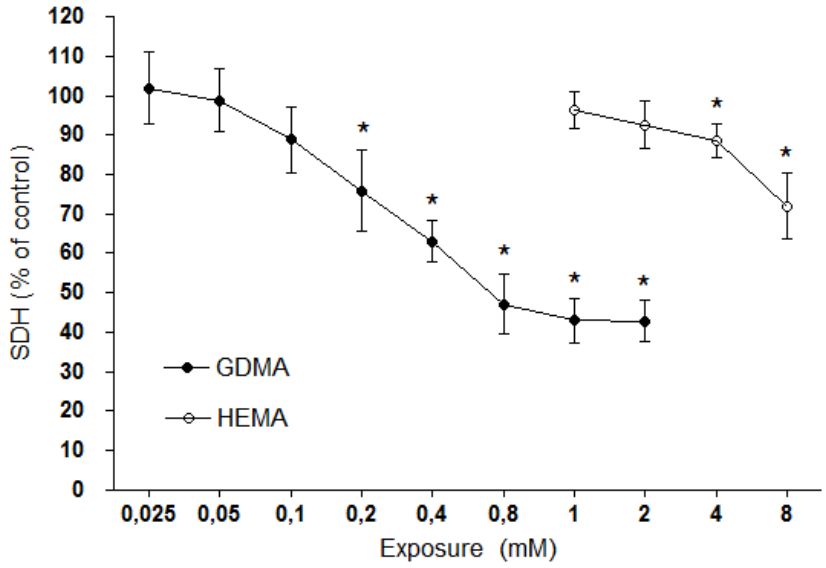

Figure 11. Cell viability in BEAS-2B cells after 24 hours of exposure to GDMA and HEMA, estimated by succinate–dehydrogenase (SDH)-activity. Control level was set to 100 %. The results are shown as mean ± S.D and N = 4. * indicates significant change from control ($p < 0.05$).

4.2 Cell death

Fluorescence microscopy of cells stained with Hoechst 33342 and propidium iodide (PI) was used to evaluate apoptosis and necrosis after 24 hours of exposure.

GDMA exposed cells showed significant apoptosis at 0.2 mM (1.3 ± 0.3 %) and 0.4 mM (2.7 ± 0.8 %) compared to control (0.5 ± 0.5 %). The highest concentrations of GDMA (0.8 mM and 1 mM) showed an increase in both apoptotic and necrotic cells, but the results were not significant.

Exposure to 4 mM HEMA gave a significant increase in apoptotic cells (2.2 ± 1 %), while 8 mM HEMA gave a significant increase in both apoptotic- (4.6 ± 0.8 %) and necrotic cells (9.2 ± 3.2 %) compared to control (0.5 ± 0.5 %) and (2 ± 1.7 %) respectively.

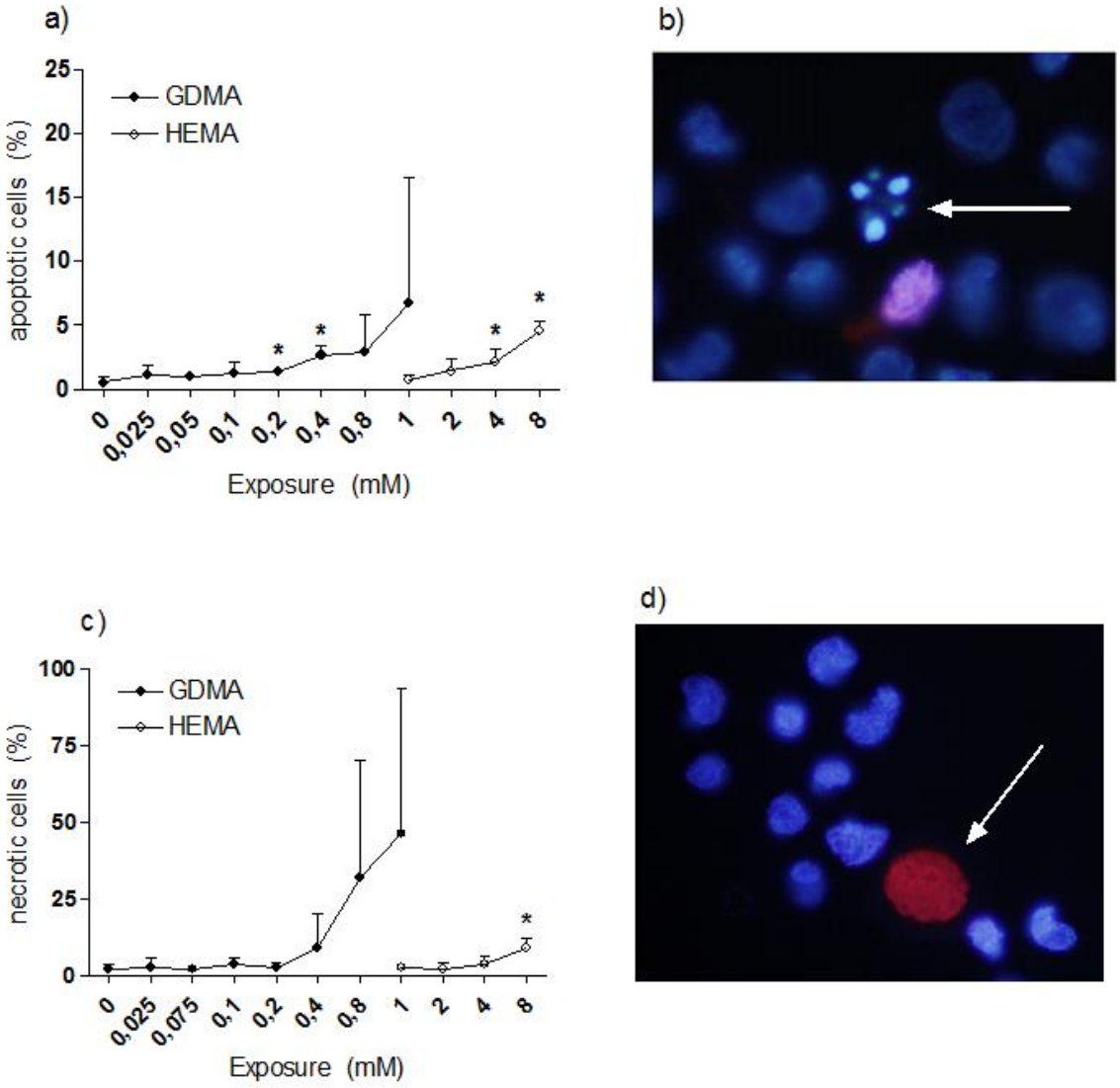

Figure 12. Apoptosis (a) and necrosis (c) induced in BEAS-2B cells after 24 hours of exposure to GDMA and HEMA, evaluated by fluorescence microscopy of cells stained with Hoechst 33342 and PI. Representative pictures from microscopy of an apoptotic cell (b) and a necrotic cell (d) are shown. Results are presented as mean ± S.D. and N = 4. * indicates significant change from control ($p < 0.05$).

Flow cytometric analysis of cells stained with annexin V and PI was used to evaluate apoptosis and necrosis after 24 hours of exposure to GDMA and HEMA. Significant apoptosis was observed after exposure to 0.4 mM GDMA (10.1 ± 1.4 %) compared to control (6 ± 2.8 %). No significant necrosis was seen following GDMA exposure. After HEMA exposure, there was no significant increase in apoptotic- or necrotic cell.

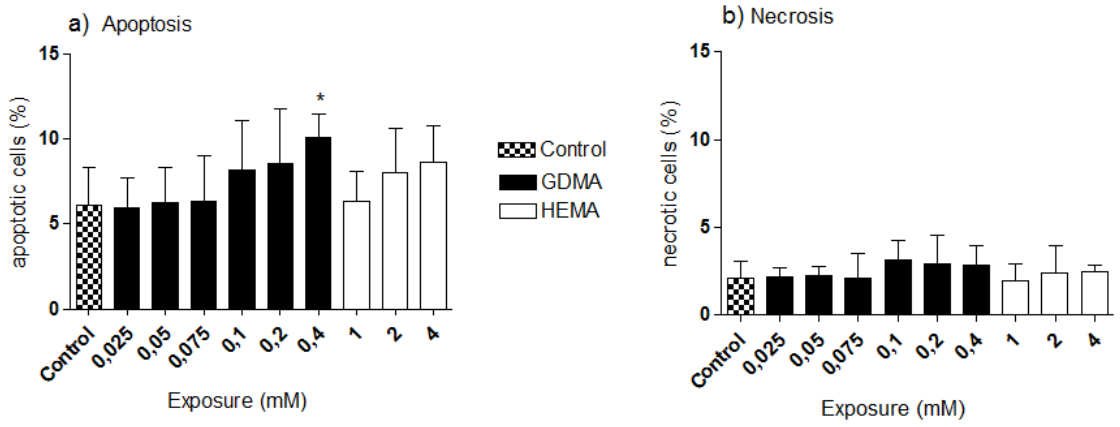

Figure 13. Apoptosis (a) and necrosis (b) in BEAS-2B cells induced by 24 hours of exposure to GDMA and HEMA, measured by flow cytometry of cells stained with annexin V and PI. The results are shown as mean ± S.D. and N ≥ 3. * indicates significant change from control ($p < 0.05$).

4.3 Glutathione (GSH)

Flow cytometric analysis of monobromobimane treated cells was used as a measurement of the GSH levels in cells after 24 hours of exposure to GDMA, HEMA, DEM and BSO.

GSH levels in cells exposed to 0.4 mM GDMA were significantly lower than control (set to 100 %) after 2 hours (22.5 ± 21 %), 6 hours (20 ± 12.9 %) and 24 hours (56.9 ± 2.9 %). Significantly reduced GSH levels were seen in cell exposed to 4 mM HEMA for 2 hours (25.6 ± 18.8 %) and 6 hours (17.8 ± 33.3 %).

Exposure to DEM gave no significant reduction in GSH levels, but after 24 hours, a significant increase was seen with all the concentrations tested. BSO reduced the level of GSH after 6 hours of exposure to - 0.01 mM (82.6 ± 4 %) and 0.025 mM (84.1 ± 3.6 %). A further reduction was seen after 24 hours, but the results were not significant.

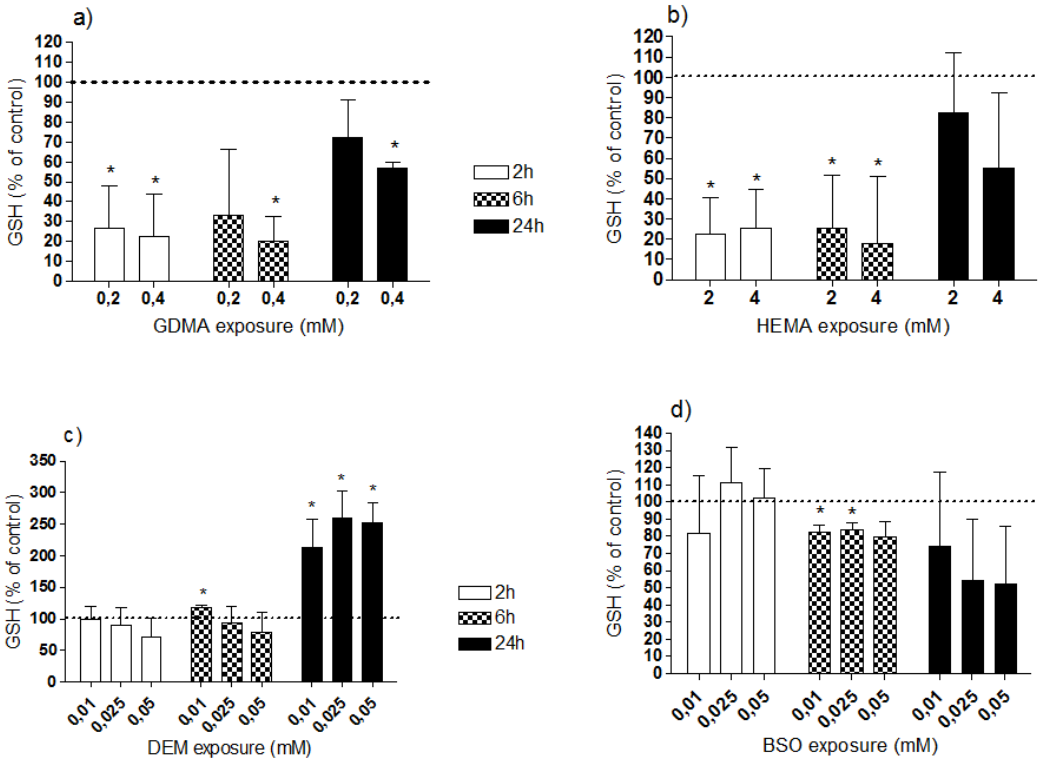

Figure 14. GSH levels in BEAS-2B cells after 2,6 and 24 hours of exposure to GDMA (a), HEMA (b), DEM (c) and BSO (d). The results are shown as mean ± S.D and N ≥ 3. Control level is set to 100 %. * indicates significant change from control ($p < 0.05$).

4.4 Reactive oxygen species (ROS)

Flow cytometric analysis of cells treated with DCFH-DA was used to measure ROS levels in cells after 24 hours of exposure to GDMA, HEMA, DEM and BSO.

ROS levels in cells exposed to GDMA were higher than control (set to 100 %), although not significant, after 2 hours. After 6 hours a significant increase in ROS was measured in cells exposed to 0.2 mM (121.2 ± 4.1 %) and 0.4 mM (124.1 ± 3.2 %) GDMA.
HEMA induced significant increase in ROS levels after 2 hours of exposure to 2 mM (140.3 ± 10.3 %) and 4 mM (144.8 ± 19 %). Significant increase in ROS levels was also seen 6 hours after exposure to 2 mM HEMA (119.8 ± 2.5 %).

No significant increase in ROS levels were seen after exposure to DEM. After 24 hours, a significant decrease was observed in cells exposed to 0.025 mM (69.1 ± 9.9 %) and 0.05 mM (74.2 ± 6 %) DEM. Cells exposed to BSO showed no significant change in ROS levels. An increase was observed after 6 hours, but the results were not significant.

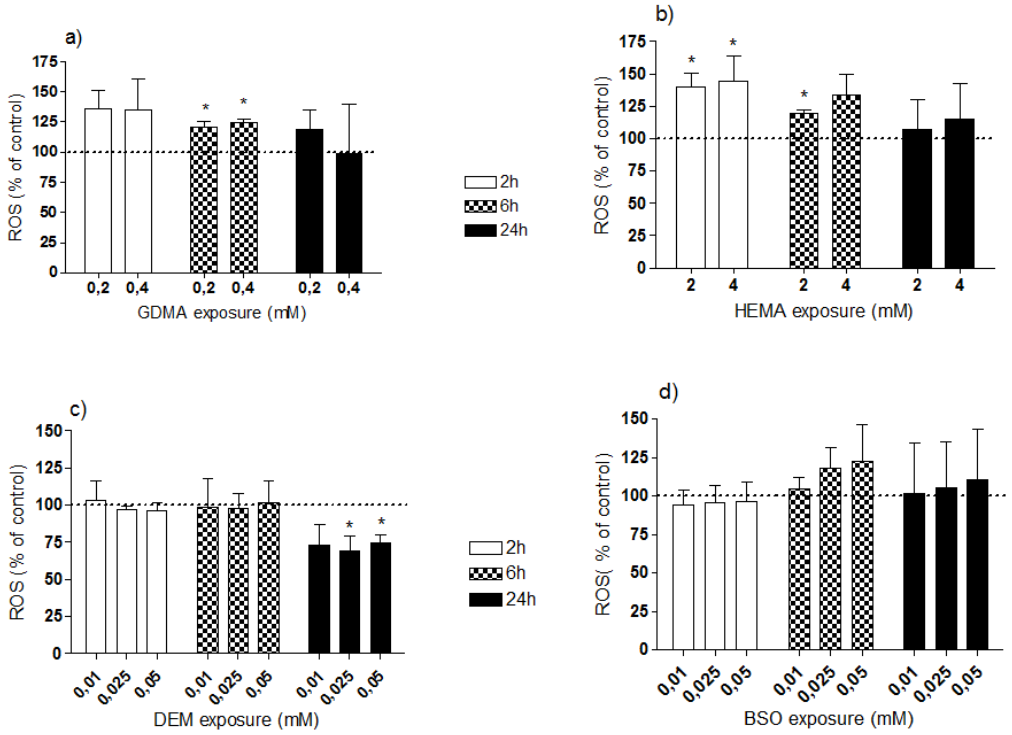

Figure 15. ROS levels in BEAS-2B cells after 2,6 and 24 hours of exposure to GDMA (a), HEMA (b), DEM (c) and BSO (d). The results are shown as mean ± S.D and N = 3. Control level is set to 100 %. * indicates significant change from control ($p < 0.05$).

4.5 Cell cycle

Flow cytometric measurements of DNA contents were used to analyse cell cycle progression in cells exposed 24 hours to GDMA, HEMA, DEM and BSO.

Significant increase in the percentage of cells in S-phase was observed after exposure to both GDMA and HEMA. Compared to control (32.7 ± 3.2 %) the percentage of cells in S-phase increased to 39.7 ± 5.7 % after exposure to 0.4 mM GDMA and 52 ± 4.6 % after exposure to 4 mM HEMA. Histograms of cell cycle distribution show that HEMA induces an accumulation of cells in early S-phase, while GDMA exposed cells are evenly distributed throughout S-phase.

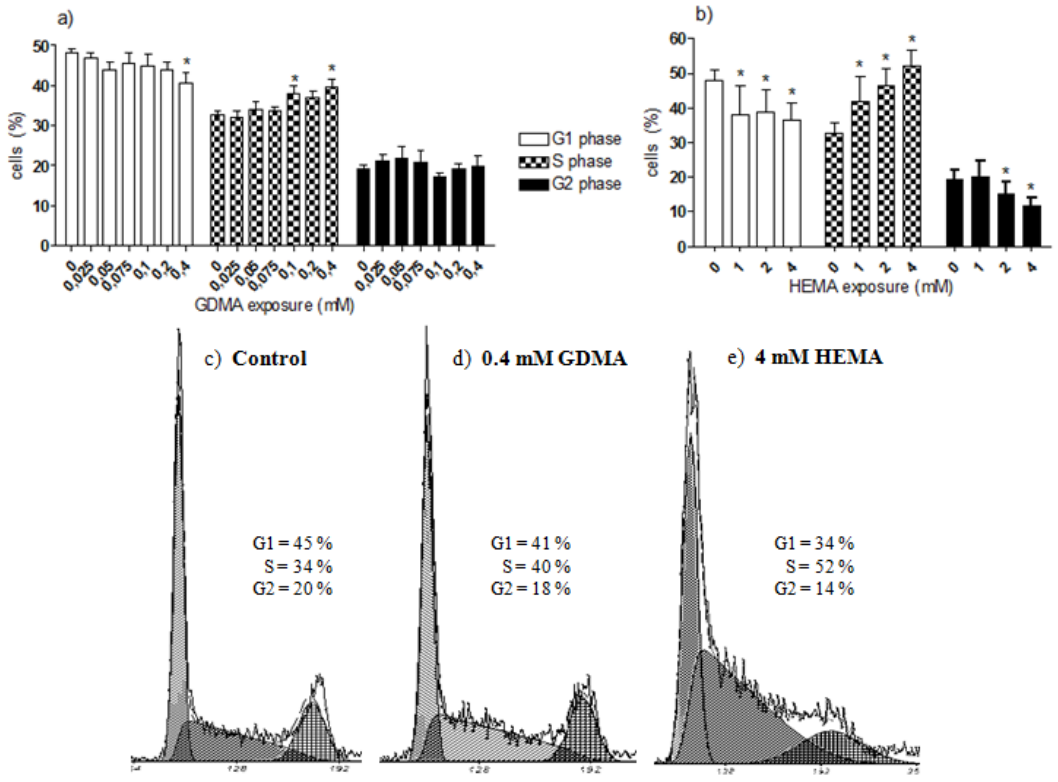

Figure 16. Percentage of BEAS-2B cells in G1-, S- and G2-phase after 24 hours of exposure to GDMA (a) and HEMA (b). The results are shown as mean ± S.D. and N ≥ 5. * indicates significant change from the control ($p < 0.05$). Representative histograms are shown for control (c), 0.4 mM GDMA (d) and 4 mM HEMA (e).

Exposure to diethyl maleate (DEM) and buthionine sulfoximine (BSO) induced no significant change in the percentage of cells in any of the phases of the cell cycle. No apparent change was seen in the histograms (data not shown).

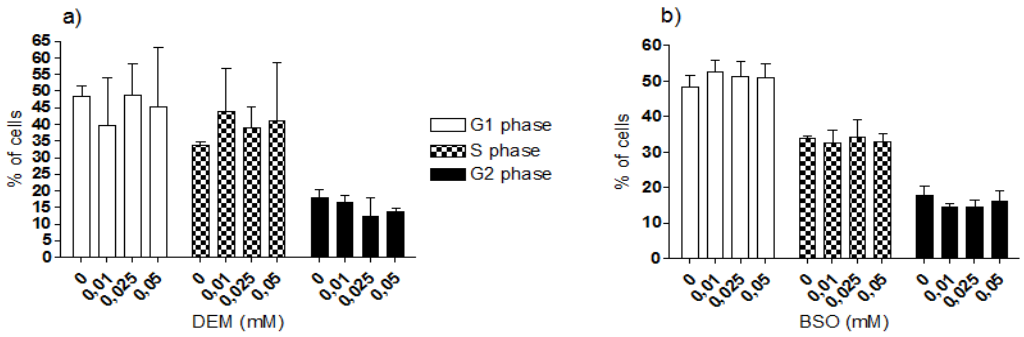

Figure 17. Percentage of BEAS-2B cells in G1-, S- and G2-phase after 24 hours of exposure to DEM (a) and BSO (b). The results are shown as mean ± S.D. and N = 3.

4.5.1 Cell cycle regulatory proteins

Western blotting was used as a technique to measure cyclin E1 and cyclin D1 levels in cells exposed 24 hours to GDMA and HEMA. β-actin and Ponceau S were used as loading controls, and showed even distribution of proteins in each lane of each experiment (data not shown).

Cells exposed to 0.4 mM GDMA showed significantly lower levels of cyclin E1 (57 ± 20 %) and cyclin D1 (6 ± 5 %) compared to control (set to 100 %). Exposure to 1 mM HEMA significantly increased the level of cyclin E1 (133.8 ± 31.6 %) and 4 mM of HEMA decreased the level of cyclin D1 (44 ± 22 %).

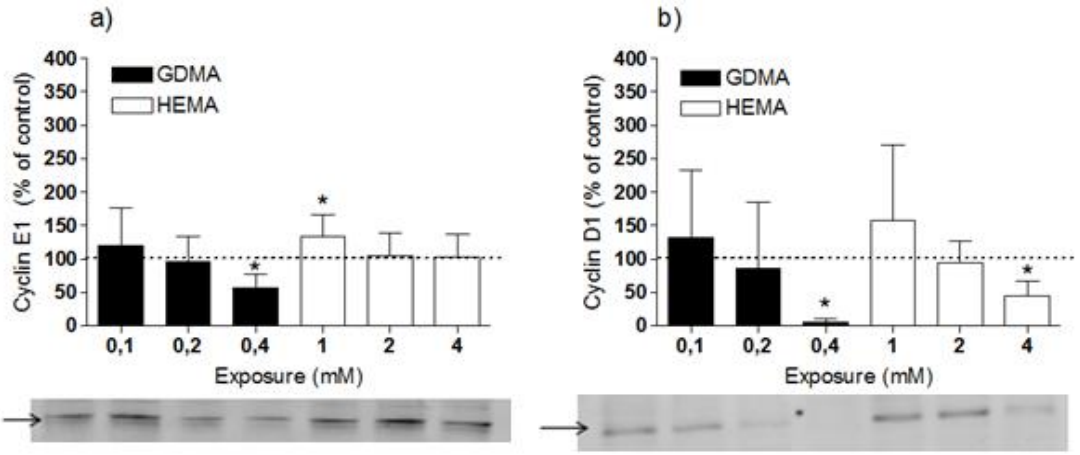

Figure 18. Cyclin E1 (a) and Cyclin D1 (b) levels in BEAS-2B cells after 24 hours of exposure to GDMA and HEMA. Representative blots are shown below. The results are shown as mean ± S.D and N ≥ 5. Dotted line illustrates the control levels (set to 100%). * indicates significant change from control ($p < 0.05$). β-actin and ponceau S were used as loading controls.

4.6 DNA damage response signalling proteins

Western blotting was used as a technique to measure levels of the DNA damage response signalling proteins pP53, γH2AX and pChk2 in cells after 24 hours of exposure to GDMA and HEMA. B-actin and Ponceau S were used as loading controls, and showed even distribution of proteins in each lane of each experiment (data not shown).

4.6.1 pP53

No change in pP53 levels were seen in cells exposed to GDMA. After exposure to HEMA, a significant increase in pP53 levels were seen at 4 mM (175.5 ± 26.7 %) compared to control (set to 100 %).

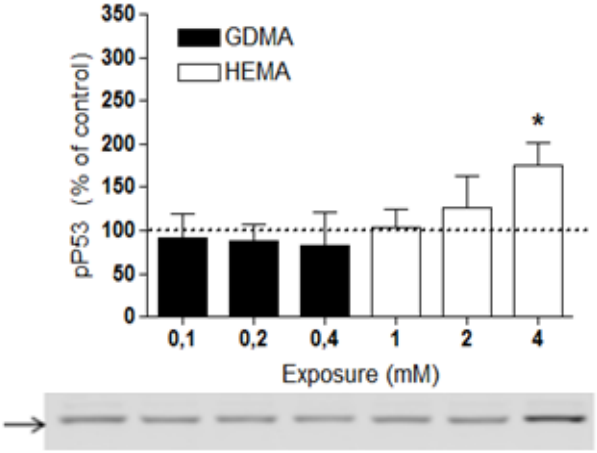

Figure 19. pP53 levels in BEAS-2B cells exposed 24 hours to GDMA and HEMA. The results are shown as mean ± S.D and N = 4. Dotted line illustrates control level (set to 100 %). A representative blot is shown below. * indicates significant change from control ($p < 0.05$). β-actin and ponceau S were used as loading controls.

4.6.2 γH2AX

Cells exposed to 0.4 mM GDMA showed a significant increase in γH2AX levels (213.3 ± 49.6 %) compared to control (set to 100 %). No change in γH2AX levels were seen in cells after HEMA exposure.

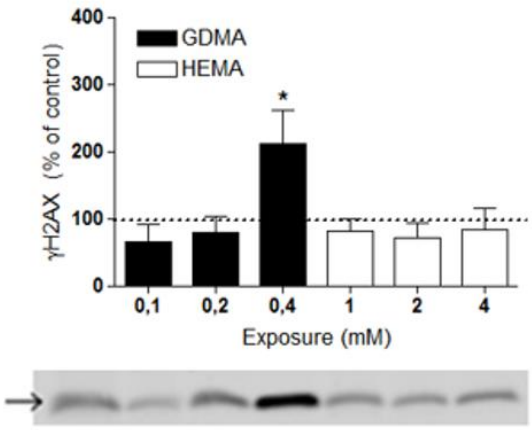

Figure 20. γH2AX levels in BEAS-2B cells exposed 24 hours to GDMA and HEMA. The results are shown as mean ± S.D and N = 4. Dotted line illustrates control level (set to 100 %). A representative blot is shown below. * indicates significant change from control ($p < 0.05$). β-actin and ponceau S were used as loading controls.

4.6.3 pCHk2

No change in pChk2 levels were seen in cells exposed to GDMA or HEMA. All the experiments gave however a level of pChk2 that was higher than control in the cells exposed to 4 mM HEMA (112 – 426 % of the control, N = 5). This was not seen after GDMA exposure.

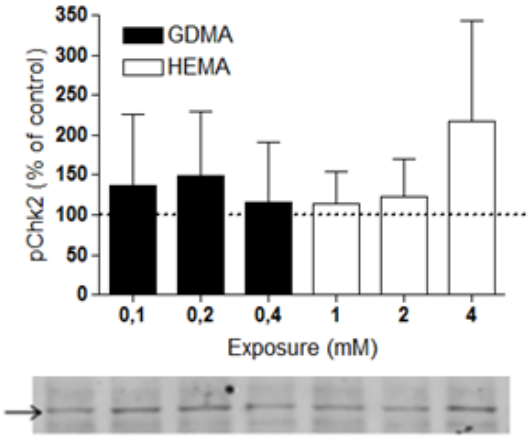

Figure 21. pChk2 levels in BEAS-2B cells exposed 24 hours to GDMA and HEMA. The results are shown as mean ± S.D and N = 5. Dotted line illustrates control level (set to 100 %). A representative blot is shown below. β-actin and ponceau S were used as loading controls.

5 Discussion

An *in vitro* model system was used in the present study to investigate and compare the cellular mechanisms following exposure to the methacrylate monomers GDMA and HEMA. The role of glutathione depletion in monomer induced cell growth disturbance was investigated comparing the effects of monomers to DEM and BSO exposure. DEM and BSO, two well-known glutathione depleting agents. By measuring levels of cyclins (D1 and E1) and activation of p53, H2AX and Chk2, the DNA damage response-signalling pathways were compared between the monomers.

5.1 Methodological considerations

Methacrylate monomers are found to be airborne in dental practices [15, 16], making airway exposure likely. Hence, BEAS-2B cells (bronchial epithelial cells) are a relevant model for studying methacrylate effects. BEAS-2B cells are commercially available and often used as an *in vitro* model for testing inhalation toxicity [41, 57, 58]. In general, immortalized cell lines are stable model systems, easy to work with and cost-effective. Therefore, these model systems are important in toxicology studies focusing on cellular response mechanisms. It is, however, important to be aware of limitations associated with an *in vitro* approach compared to the *in vivo* situation. Cell lines are homogenous and cannot mimic the complexity of an organism where multiple cells and factors constantly interact. In addition, cell lines have altered properties compared to their *in vivo* counterpart. Based on this, extrapolation from *in vitro* studies to the *in vivo* situation must be done with caution. *In vitro* systems are, however, clearly valuable in early cellular response studies that may form the basis for further *in vivo* research [59].

The monomer concentrations used in this study are relatively high compared to concentrations measured in salvia of patients after treatments [13, 17, 60] and in the air of dental practices [15, 16]. However, the exposure period in a clinical situation is extended compared to what is achievable in an *in vitro* system. To induce cellular responses *in vitro*, higher concentrations are

needed. The exposure concentrations chosen are below the level where extensive cell death are induced, to avoid interfering signalling from DNA degradation during cell death [61].

Cell viability was estimated using the MTT assay, which actually measures the SDH-enzyme activity in mitochondria [45]. This activity can be reduced due to cell death, but also reduced proliferation and impaired mitochondrial activity. The MTT assay was therefore supplemented with fluorescence microscopy and flow cytometry to detect cell death and changes in cell proliferation. Fluorescence microscopy allows visual detection of apoptotic and necrotic cells. The relatively low number of cells that can be assessed with this method is, however, a disadvantage. Flow cytometry lack the advantage of visualization, but allows a large amount of individual cells to be analysed quickly and with high precision [20]. In combination, these methods provide a thorough assessment of cytotoxicity.

Western blotting was used to detect and quantify protein levels. The use of loading controls verifies even amounts of protein in each sample. Through electrophoresis, the proteins are separated based on molecular weight. This will prohibit that unspecific binding of antibodies interferes with the results, which can be a weakness of other immunoassays such as ELISA. Western blotting has been considered "semi-quantitative" due to limitations in the detection step. This limitation has been eliminated due to the use of direct scanning and imaging software, which provides a much wider linear dynamic range compared to the classical techniques.

5.2 Discussion of results

Both GDMA and HEMA reduced the number of viable BEAS-2B cells in a concentration-dependent manner. Increased apoptotic cell death and induced changes in the cell growth pattern were observed, in line with previous findings [35, 36, 38, 39, 41, 62]. The aim of this study was, however, to elucidate differences in cellular response mechanisms. GDMA appeared to be more potent than HEMA, as a lower concentration of GDMA was needed to significantly reduce cell viability. This difference is also observed in previous findings [10, 35]. A 10-fold difference in potency to induce cell death was observed between GDMA and HEMA. Therefore, when comparing cellular response mechanisms 0.4 mM GDMA were compared to 4 mM HEMA.

In line with other studies, GDMA and HEMA induced changes in the cell growth pattern [38, 39, 41, 62]. However, appearance of the cell cycle histograms differs between the two methacrylates. HEMA exposed cells show accumulation in early S-phase. In contrast, the increase after GDMA exposure was evenly distributed throughout the S-phase. Stalled cell cycle progression allows activation of DNA damage repair, protecting cells from copying damaged DNA [63, 64]. The observed effects on cell cycle distribution could therefore be caused by DNA damage and activation of repair mechanisms induced by GDMA and HEMA. In addition to inducing accumulation of cells in S-phase, GDMA and HEMA significantly changed cyclin levels in BEAS-2B cells. Cyclins in complex with cyclin-dependent kinases (CDKs) are essential in regulating the progression through the cell cycle. GDMA and HEMA had different effects on cyclin levels in BEAS-2B cells. Cyclin D1 levels were reduced after exposure to both monomers. Levels of cyclin E1 on the other hand, were reduced by GDMA, but not by HEMA. Interestingly, the lowest concentration of HEMA increased the cyclin E1 levels. Overexpression of these cyclins are associated with accelerated entry into S-phase [65]. Cyclin D1 and E1 are involved in phosphorylation of the retinoblastoma protein (Rb) and activation the transcription factor E2F, initiating DNA synthesis [66]. Hence, down regulation of cyclins can possibly delay the entry into and the progression through S-phase, due to inhibition of DNA synthesis.

Taken together, the effects on cell cycle distribution and cyclin levels support involvement of DNA damage. However, the mechanisms seem to differ between GDMA and HEMA.

Activation of DNA damage signalling proteins in BEAS-2B cells indicates induction of DNA damage by both GDMA and HEMA. DNA double strand breaks (DSBs) are detected by the protein kinase ATM, which further activates other signalling proteins, including H2AX and Chk2 [27, 30, 67]. The phosphorylation of H2AX is established as a specific and sensitive molecular marker of DSBs [68]. The DNA damage response caused by HEMA is suggested to involve P53, H2AX and Chk2 [36, 39, 41]. The complex role of p53 in cell signalling makes simplification of its role difficult, but DNA damage is one of the stressors able to activate p53 [20]. Both cell cycle arrest and apoptosis are possible endpoints in p53 signalling pathways [69]. In the current study, HEMA increased activation of P53 and Chk2, but not H2AX. GDMA induced activation of H2AX, but not p53 or Chk2. GDMA is previously shown to induce DSBs *in vitro*, determined by the γH2AX assay [70]. DSBs caused by the apoptotic process are also able to activate H2AX

[61]. However, the lack of H2AX activation in cells exposed to 4 mM HEMA suggests that DSBs are due to direct DNA damage. Activation of Chk2 by GDMA and H2AX by HEMA cannot be excluded, as the measurements were conducted at only one time point. Previous studies have measured H2AX activation after HEMA exposure [41].

DNA damage caused by methacrylate monomers is suggested, at least in part, to be a result of oxidative stress [36, 71]. Depletion of glutathione (GSH) and an increase in reactive oxygen species (ROS) is a well-documented response to methacrylate monomers [36]. HEMA induced cell death was increased when cells were GSH-depleted prior to HEMA exposure, illustrating the importance of GSH for cellular defence [42]. In the current study, comparable concentrations of GDMA and HEMA induced similar oxidative stress response in BEAS-2B cells. However, growth patterns were affected differently, suggesting involvement of additional mechanisms.

DEM and BSO were included to investigate if the cell cycle disturbance after monomer exposure could be explained by GSH depletion and oxidative stress. Reduction of GSH levels were seen six- and twenty-four hours after BSO exposure in BEAS-2B cells, while DEM did not induce detectable GSH-depletion. The cells respond to DEM exposure by increasing its GSH-production. This could indicate adaption to GSH-depletion not detectable by the method used in the current study. In other cell lines, reduction of cell proliferation is previously implied for both DEM [72] and BSO [73]. DEM is believed to deplete nuclear GSH [72], while BSO inhibits GSH synthesis [74]. Nuclear GSH could therefore be important in cell cycle progression as DEM, and not BSO, led to impaired cell proliferation of 3T3 fibroblasts [72]. In the current study, however, cell growth disturbance could not be observed after exposure to DEM and BSO. Overall, this indicate different involvement of GSH in growth regulation in different cell lines.

6 Conclusions

In the current study, GDMA and HEMA induced cytotoxic responses in BEAS-2B cells. Similar changes in GSH and ROS levels were observed after exposure to both monomers. Evidence of DNA damage was also observed, but the DNA damage response differed between the two monomers. Hence, GSH-depletion and ROS increase alone cannot explain methacrylate induced toxicity in BEAS-2B cells.

Future perspectives

The work presented in this thesis show involvement of different mechanisms in the *in vitro* methacrylate induced DNA damage response. This raises questions relevant for future studies.

Studies on other DNA damage signalling response pathways would be valuable, especially related to GDMA induced genotoxicity. The signalling proteins ATR and Chk1, associated with single strand breaks could be relevant for future work.

Possible effects on the retinoblastoma protein (Rb) and the transcription factor E2F could be interesting for further investigations of methacrylate induced cell cycle regulation. In addition, as nuclear GSH content were suggested relevant for cell cycle progression, it would be useful to obtain insights into the GSH distribution in different cellular compartments.

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